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Effect of Organic Acids and Hydrogen Peroxide on *Cryptosporidium parvum* Viability in Fruit Juices

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ABSTRACT

Cryptosporidium parvum has historically been associated with waterborne outbreaks of diarrheal illness. Foodborne cryptosporidiosis has been associated with unpasteurized apple cider. Infectious oocysts are shed in the feces of common ruminants like cattle and deer in and near orchards. In this study, the ability of organic acids and hydrogen peroxide (H_2O_2) added to fruit juice to inhibit the survival of *C. parvum* was analyzed. Oocyst viability was analyzed by a cell culture infectivity assay with the use of a human ileocecal cell line (HCT-8) whose infectivity pattern is similar to that for human oral infectivity. Cell monolayers were infected with 10^6 treated oocysts or a series of 10-fold dilutions. Parasitic life stages were visualized through immunohistochemistry with 100 microscope fields per monolayer being counted. In vitro excystation assays were also used to evaluate these treatments. Organic acids and H_2O_2 were added to apple cider, orange juice, and grape juices on a weight/volume basis. Malic, citric, and tartaric acids at concentrations of 1 to 5% inhibited *C. parvum*'s infectivity of HCT-8 cells by up to 88%. Concentrations ranging from 0.025 to 3% H_2O_2 were evaluated. The addition of 0.025% H_2O_2 to each juice resulted in a >5-log reduction of *C. parvum* infectivity as determined with a most-probable-number–based cell culture infectivity assay. As observed with differential interference contrast and scanning electron microscopy, reduced infectivity may be mediated through effects on the oocyst wall that are caused by the action of H_2O_2 or related oxygen radicals. The addition of low concentrations of H_2O_2 can represent a valuable alternative to pasteurization.

Cryptosporidium parvum causes a gastrointestinal illness that can vary from a self-limiting diarrhea to a severe wasting disease *(37).* Cryptosporidiosis may persist in immunocompromised persons, especially those who have AIDS or are undergoing chemotherapy *(8),* and in malnourished children *(18).* Cryptosporidiosis is associated with waterborne outbreaks and is becoming more recognized as a cause of foodborne illness *(14).* Within the past 10 years, several outbreaks of gastrointestinal illness have been associated with fresh fruit juice and cider *(1, 30).* Most juice outbreaks have been caused by pathogenic bacteria, such as *Escherichia coli* O157:H7 and *Salmonella* serovars, while at least two outbreaks have been attributed to *C. parvum (5, 27).*

The life cycle of *C. parvum* begins with the ingestion of the environmentally resistant oocyst, which excysts within the host intestine. Infective sporozoites enter microvilli of enterocytes and replicate there until oocysts are formed and are shed in the feces *(13). Cryptosporidium* oocysts are fairly resistant to most chemical compounds because of their relative metabolic dormancy and impermeability of their oocyst walls *(22).* Organic acids such as citric, malic, and tartaric acids exhibit antimicrobial activity and have been used in many food applications *(10).* It is believed that organic acids act on microbial cells by lowering the pH of the environment, thus disrupting the functions of membranes and key enzymes *(11).* Unlike bacteria, the environmentally resistant oocyst can resist changes in pH. The components of pH ($H+$ and $OH-$) are charged and remain outside the oocyst wall, where they alone cannot inactivate oocysts *(23).* Pretreatment with acidified media has previously been shown to enhance excystation indirectly, presumably by increasing oocyst wall permeability *(24).* Additionally, the ability of some organic acids to chelate metal ions like calcium (Ca^{2+}) and magnesium (Mg^{2+}) may increase the effectiveness of these acids in inhibiting oocyst viability, since these ions are essential for oocyst maintenance.

In accordance with the fruit juice hazard analysis critical control point rule *(1),* treatment of the final juice product is not limited to heat pasteurization. Alternatives to pasteurization may be implemented in juice processing if they provide a 5-log reduction of the pertinent pathogen. Various nonthermal alternatives exist. One alternative may be the addition of H_2O_2, the most widely used of the inorganic peroxides, which exerts a strong antimicrobial effect through the production of reactive oxygen species like hydroxyl radicals *(3, 10).* Hydrogen peroxide gas plasma at

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58%, used as a medical sterilant, has previously been shown to have an inhibitory effect on *C. parvum* oocysts *(39).* Additionally, treatment with H_2O_2 was shown to increase the permeability of the oocyst wall, altering the acid-fast staining pattern *(12).*

Treatments involving changes in pH or oxidation affect oocysts in different ways that can be gauged by excystation and infection assays. For example, oocysts that have been killed by heat or formaldehyde will not excyst, demonstrating that live sporozoites may be required for excystation *(22, 24).* It is possible that some treatments for which no developmental life stages are detected do not fully inhibit excystation but significantly damage sporozoite DNA. It has also been noted that treatments that kill sporozoites may induce changes within the oocyst wall and suture, thereby inhibiting excystation to some degree *(24).*

The objectives of this study were to identify the minimum levels of H_2O_2 and organic acids that must be added to fruit juices to reduce or inhibit the infectivity of *C. parvum.* An HCT-8 cell culture infectivity assay *(31)* was used to evaluate the amended fruit juice samples with regard to oocyst excystation and sporozoite viability. This assay provides information on the inhibition of *C. parvum* viability as a function of treatment concentration. Cell culture is an excellent tool for studying parasite development, and the infection pattern for the cell type used in this assay (HCT-8) most closely mimics human infection *(6).* In vitro excystation assays were used to further evaluate the effects of juice treatments on oocyst wall structure, suture condition, and sporozoite viability.

MATERIALS AND METHODS

Oocyst preparation. *C. parvum* oocysts (bovine Beltsville isolate, genotype C) were originally obtained from infected dairy calves and processed at the Animal Waste Pathogen Laboratory, U.S. Department of Agriculture, Beltsville, Md. *(16).* Oocysts were purified from fecal material first by washing through a series of graded sieves down to a pore size of 45 μm and then by density centrifugation over cesium chloride *(25).* Residual cesium chloride was removed with three 10-min cycles of centrifugation at 1,000 × *g.* Oocysts were resuspended in distilled water and quantified on a hemocytometer.

Oocyst treatment. *C. parvum* oocysts (1×10^6 oocysts per treatment) were treated with 0.525% sodium hypochlorite at 4°C for <5 min and washed twice with Hanks balanced salt solution (HBSS). The oocysts were then incubated with 1.5 ml of treated or untreated fruit juice at 4°C for a specified period and washed twice with HBSS after treatment. Oocyst recovery levels were 97 to 100% after each wash. If juice pulp (apple cider and orange juice) was present in the juice, it was pelleted along with the oocysts but did not interfere with oocyst infection or excystation. Treatment with sodium hypochlorite did not affect juice treatment (data not shown). No difference between oocysts treated in juice incubated at 4°C and those treated in juice incubated at 25°C was observed (data not shown). Various levels of food-grade H_2O_2 (FMC, Philadelphia, Pa.) and organic acids (Presque Isle Wine Cellars, North East, Pa.) were added to apple cider, orange juice, purple grape juice, and white grape juice on a weight/volume basis. The organic acid added corresponded to the dominant organic acid for the juice involved: malic acid for apple cider, citric acid for orange juice, and tartaric acid for grape juices. Untreated juices and oocysts treated with HBSS served as controls.

Juice preparation. Unpasteurized apple cider was obtained from a local processor, frozen, stored, and thawed at 4°C prior to being used. Orange juice (Kroger Original Premium Pasteurized Orange Juice, Kroger, Cincinnati, Ohio) was obtained from a local supermarket and stored frozen until it was used. Purple grape juice (Welch's 100% Grape Juice, Welch's, Concord, Mass.) and white grape juice (Santa Cruz Organic White Grape Juice, Santa Cruz, Chico, Calif.) were stored at room temperature before they were used. All juices were free of preservatives.

Juice analysis. Acids were added in granular form on a weight/volume basis. All acids were food-grade acids (Presque Isle). No precipitates formed when acids were added to juices. Juice samples were stored at 4°C. The levels of H_2O_2 indicated may not be exact because of dissipation, but residual H_2O_2 could not be determined (peroxide test strips, Merck, Darmstadt, Germany). pH was determined, titratable acidity (TA) was determined through 1 M NaOH titration to pH 8.2, total phenol content was determined, and color was measured with a Minolta color analyzer with the use of L*, a*, and b* values. Phenol components were measured through spectral estimation. Absorption readings were used to estimate the concentration of total phenols ($A_{280} - 4$). The concentrations of dominant organic acids (malic, citric, and tartaric acids) and sugars (sucrose, glucose, and fructose) were determined by high-pressure liquid chromatography (HPLC). Prior to HPLC, sugars and organic acids were separated and collected with the use of an anion exchange resin (BioRex, Bio-Rad, Hercules, Calif.). The fractions were then run separately, and concentrations were determined by comparison with standard sugars and acids on a Resex ROA-organic acid column with a carbohydrate guard column (Phenomenex, Torrance, Calif.) with the use of 0.01 N sulfuric acid as the mobile phase at 22°C with UV and refractive index detectors.

To establish flavor quality standards, taste evaluations of juice samples with added H_2O_2 and organic acids were carried out by the in/out method *(29).* Twenty-five untrained panelists each tasted five juice samples (control juice, juice with 0.1% H_2O_2, juice with 0.03% H_2O_2, juice with 0.3% organic acid, and juice with 0.1% organic acid) and rated these samples as either "in" or "out" of their personal specifications according to their individual perceptions of flavor quality. If a juice was deemed "out," taste descriptors were requested. A minimum of 65% agreement was required for a particular juice to be considered "in." Unpasteurized apple cider and orange juice and pasteurized purple grape and white grape juices were used to describe changes in color and appearance as a result of the addition of H_2O_2.

Cell culture media. Human ileocecal adenocarcinoma cells (HCT-8 cells; ATCC CCL-244, American Type Culture Collection, Manassas, Va.) were maintained in RPMI 1640 medium (Mediatech Cellgro, Herndon, Va.) supplemented with L-glutamine (300 mg/liter; Mediatech Cellgro) and *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (25 mM; Mediatech Cellgro). For normal cell maintenance, medium was supplemented with 5% fetal bovine serum (Biofluids, Inc., Rockville, Md.), and the level of fetal bovine serum was increased to 10% for parasite infection (growth media) *(38).*

Cell maintenance. Stock HCT-8 cells were maintained in 75-cm^2 tissue culture flasks in a 5% CO_2 atmosphere at 37°C and at 100% humidity and passaged every 3 to 5 days. Cells were lifted from the surface with a solution of 0.25% (wt/vol) trypsin and 0.53 mM EDTA in phosphate-buffered saline (PBS) (Mediatech

Cellgro). Trypsinization required 10 to 12 min of incubation in the solution at 37°C to assist in the disruption of the cell monolayer. The cells were collected and pelleted by centrifugation for 10 min at 1,000 × *g*, resuspended in maintenance medium, and split 1:10. Cell viability was assessed with trypan blue exclusion (0.02% in PBS), and cells were counted with a hemocytometer.

Inoculation of monolayers with parasites. Collected cells were seeded on sterile 22-mm^2 glass coverslips in 6-well cluster plates (Corning, Corning, N.Y.) at 1 × 10^6 cells per well and grown to ca. 95% confluency in maintenance medium (48 h). *C. parvum* oocysts were treated as described above. For the infection of monolayers, prior to inoculation with oocysts, maintenance medium was removed and 2 to 3 ml of growth medium was added to each well in 6-well cluster plates. Cells were then incubated with treated or untreated (positive control) oocysts (10^6 oocysts in 1 ml growth medium) for 90 to 120 min. Then, each inoculated well was washed twice with HBSS to wash away unexcysted oocysts, oocyst walls, and other materials that may have been liberated from the excysted oocysts. Cells in cluster plates were then placed back in the incubator for 48 h with 3 to 4 ml of maintenance medium per well.

Immunohistochemistry of viable parasitic life stages. Parasite infection was assessed 48 h postinfection with the use of an immunohistochemistry stain *(31)*. Coverslips in 6-well cluster plates were fixed with 100% methanol for 20 to 30 min and washed twice with PBS for 5 min each time. Coverslips were removed from cluster plates and processed on slides, first with a rabbit anti–*C. parvum* primary antibody (courtesy of C. Dykstra, Auburn University) and then with a biotinylated anti-rabbit secondary antibody (Vectastain ABC Kit, Vector Laboratories, Burlingame, Calif.) and an avidin biotinylated complex (ABC reagent, Vectastain ABC Kit, Vector Laboratories). Life stages were visualized with an immunoperoxidase stain with H_2O_2 (Sigma Chemical Co., St. Louis, Mo.) and diaminobenzidine tetrahydrochloride (DAB, Sigma), with hematoxylin (Fisher Scientific) being used as a counterstain. With this system, *Cryptosporidium* life stages are colored brown on a blue-and-purple background. Oocysts that had been frozen in liquid nitrogen for 2 h prior to host cell inoculation served as a negative control. Paromomycin (500 μg/ml; Sigma) was used as a positive anti–*C. parvum* drug to determine the ability of our assay to detect reduction in developmental stages in treated cell cultures (data not shown).

Treatment effectiveness was based on the presence or absence of *C. parvum* life stages (sexual gamonts and asexual meronts) in 50 (most-probable-number [MPN]–type assays) or 100 sequential and nonoverlapping fields visualized at ×400 magnification with phase-contrast microscopy. Fields containing one or more *Cryptosporidium* life stages were scored as positive, while fields containing no stages were scored as negative. Each individual experiment was performed in triplicate. Data are shown two ways: as percentages of inhibition and as log reductions.

The percentage of inhibition relative to control untreated oocysts was calculated as [(control oocysts − treated oocysts)/control oocysts] × 100. Data are expressed as mean percentages of reduction ± standard deviations. The oocyst infectivity titer was determined for the most effective treatments by the MPN method *(34, 35)*. Calculations were based on the numbers of positive and negative fields (test units), with the Thomas equation being used to obtain the MPN. At least 100 cell culture fields were counted in three sequential dilutions with 10^6, 10^5, and 10^4 oocysts.

Excystation assays. Approximately 1 × 10^5 *C. parvum* oocysts were washed and treated as described above for use in cell culture infectivity assays. Oocysts were then incubated in 0.75% sodium taurocholate (Sigma) for 30 min at 37°C. The excystation solution was observed at ×400 and ×1,000 magnification with differential interference contrast microscopy. One hundred shells and oocysts were counted. A minimum of duplicate samples were evaluated. Oocysts containing sporozoites were considered unexcysted. Oocysts containing no sporozoites (also called shells) were considered excysted. Excystation rates were calculated by the Woodmansee method *(17, 40)*: excystation = (oocysts excysted/total oocysts counted) × 100. The log excystation reduction was determined by an adaptation of Chick's law for log inactivation based on the number of survivors (N) and the initial applied dose (N_0) *(17)*: $\log N/N_0 = \log(\%\ \text{excystation}_{\text{treated}}/\%\ \text{excystation}_{\text{control}})$.

SEM. Scanning electron microscopy (SEM) was used to evaluate oocyst surface topography after treatment with H_2O_2. *C. parvum* oocysts (ca. 5.0 × 10^5 oocysts) treated with apple cider plus 0.03% H_2O_2 for 2 h were gently mixed with HCT-8 cells (ca. 1.0 × 10^4 cells) fixed in 2.5% glutaraldehyde in 0.1 M sodium phosphate buffer and coated with gold particles for SEM. Oocysts were examined with a JOEL 35-C SEM.

Statistical analysis. Data obtained in individual experiments were considered independent. Significant differences were determined with PROC GLM of SAS statistical software (SAS Institute, Cary, N.C.). A treatment's effects on parasite viability were considered significant when $P \leq 0.05$. Treatments were separated on the basis of Tukey's test for significant differences.

RESULTS AND DISCUSSION

Characteristics of amended juice. The major sugars, acids, and phenols were evaluated in amended and unamended fruit juices, including apple cider, orange juice, and white and purple grape juices. Average pH and TA values were obtained in five experiments, and the standard deviation for all averages was <0.05. TA is expressed in milliequivalents of the dominant acid per unit of juice. For the same juice, pH and TA values were considered significantly different from one another when $P < 0.05$. It was determined that the addition of organic acids and/or H_2O_2 resulted in a significant change in acidity, as shown by pH and TA values (Table 1).

The L*, a*, and b* color values ($n = 3$) for each juice sample were determined with a Minolta Colorimeter. The magnitude of a color change in a modified juice sample compared with that in an unmodified juice sample is expressed as ΔE^*ab, which is defined as $\sqrt{(\Delta L^*)^2 + (\Delta a^*)^2 + (\Delta b^*)^2}$. The color of the juice was altered by some of the treatments, as indicated by L*, a*, and b* color values (Table 1). The ΔE^*ab value indicates a change from the unamended juice but does not indicate the direction of change (i.e., whether the juice becomes darker or lighter). In general, if the addition of H_2O_2 changed the color, the amended juice was lighter in color than the control juice.

Phenolic compounds are particularly prominent in fruits and influence a fruit's color, flavor, and antimicrobial properties. Total phenols (in absorbance units) were measured spectrophotometrically for each juice sample. Total phenols (Table 1) are expressed as gallic acid equivalents (mg/liter GAE) on the basis of the following equation: total phenolics = $29.5(A_{280}$ − 4 in absorbance units) + 210.

TABLE 1. *Changes in pH, titratable acidity (TA), total phenols, and color values for juice samples*[a]

Sample	pH	TA (meq acid)	Phenol concn (mg/liter GAE)	ΔE*ab
Apple cider	3.90	0.39	1,239.55	N/A
+ 3% malic acid	2.90	3.23	1,180.55	1.07
+ 5% malic acid	2.43	4.91	897.35	0.82
+ 3% H_2O_2	3.78	0.22	1,266.10	1.64
+ 1% malic acid + 1.5% H_2O_2	3.62	1.22	1,230.70	1.54
+ 0.03% H_2O_2	3.90	0.36	1,236.66	0.15
Orange juice	3.82	0.63	1,481.45	N/A
+ 3% citric acid	2.88	3.56	1,331.00	0.54
+ 5% citric acid	2.76	5.27	1,213.00	0.92
+ 3% H_2O_2	3.74	0.51	1,245.45	1.07
+ 1% citric acid + 1.5% H_2O_2	3.20	1.66	1,221.85	1.03
+ 0.03% H_2O_2	3.89	0.62	1,404.75	0.10
White grape juice	3.14	0.47	1,183.50	N/A
+ 3% tartaric acid	2.33	3.44	1,239.55	1.77
+ 5% tartaric acid	2.20	4.89	1,561.10	1.60
+ 3% H_2O_2	3.08	0.61	1,171.70	3.85
+ 1% tartaric acid + 1.5% H_2O_2	2.50	1.43	1,419.50	1.76
+ 0.03% H_2O_2	3.10	0.44	1,272.00	0.10
Purple grape juice	3.41	0.52	1,295.60	N/A
+ 3% tartaric acid	2.54	3.43	1,254.30	0.22
+ 5% tartaric acid	2.30	5.21	1,207.10	0.27
+ 3% H_2O_2	3.36	0.62	1,215.95	2.02
+ 1% tartaric acid + 1.5% H_2O_2	2.80	1.40	1,198.25	1.15
+ 0.03% H_2O_2	3.39	0.54	1,272.00	0.65

[a] Values for pH, TA, and phenol concentration are averages for five experiments (standard deviation < 0.05). Color change values (ΔE*ab) were obtained from three experiments.

Liquid chromatography was used to identify dominant sugars and acids. No significant differences between amended and control juices with respect to sucrose, glucose, or fructose levels were observed ($P < 0.05$; data not shown). Significant differences between the levels of dominant juice acids (malic, citric, and tartaric acids) were detected only for acidified samples ($P < 0.05$). The addition of organic acids and/or H_2O_2 caused significant changes in the phenol components of a juice. All juices to which 5% acid had been added were significantly different from control juices in this respect, while the addition of 0.03% H_2O_2 did not significantly alter juices' phenol components compared with those of controls ($P < 0.05$). Not all levels of added H_2O_2 and organic acids were evaluated in juices, but the higher levels were evaluated. Lower levels were found to result in less extensive changes (data not shown). Overall, these observed changes in juice characteristics did not seem to affect the viability of *Cryptosporidium* oocysts directly. Rather, a loss in viability appears to be a result of accumulated weakening of membranes by free radicals, which arises from the addition of H_2O_2 and/or relatively large amounts of organic acid.

Effects of organic acids on infectivity. Cell infection and parasite reproduction were evident on the basis of life stages recognized by the *C. parvum* polyclonal antibody (Fig. 1). Life stages were additionally confirmed by transmission electron microscopy *(31)*. Infection in HCT-8 cells by *C. parvum* oocysts incubated in fruit juices acidified with the addition of the dominant organic acid was evaluated and compared with infection by untreated oocysts incubated in HBSS. Figure 2 shows the inhibition of *C. parvum* infectivity in untreated apple cider and in apple cider acidified with 1, 3, and 5% malic acid (Fig. 2A); in untreated orange juice and in orange juice acidified with 1, 3, and 5% citric acid (Fig. 2B); in untreated purple grape juice and in purple grape juice acidified with 1, 3, and 5% tartaric acid (Fig. 2C), and in untreated white grape juice and in white grape juice acidified with 1, 3, and 5% tartaric acid (Fig. 2D). All graphs show the general trend of an increase in the inhibition of infectivity with an increase in the level of added acid and a resulting decrease in pH. The acid concentrations used were not sufficient to completely inhibit *C. parvum* viability, and the acidified juices were not palatable, as determined by a sensory evaluation whose results indicated that juice with 0.3% acid added was considered out of the personal specifications of >65% of the panelists (data not shown) *(29)*. The addition of 1 or 3% acid did not result in a significant difference for any juice, while juices with 5% acid added were significantly different from unamended juices with respect to infectivity with the exception of white grape juice, for which no differences in infectivity between samples were detected. As anticipated, a change in pH was not completely effective in inhibiting the infectivity of *C. parvum*. Preincubation in acids has

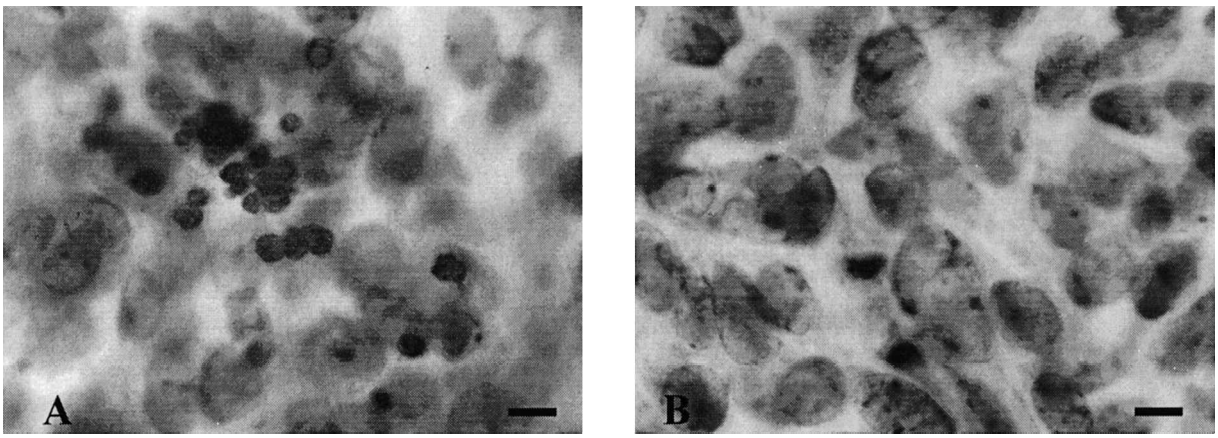

FIGURE 1. *Cell culture infectivity. HCT-8 cell monolayers were infected with 1 × 10⁶ untreated oocysts (A) or with 1 × 10⁶ oocysts frozen in liquid nitrogen (B). Sexual meront and asexual gamont life stages are observed with an immunohistochemistry stain with hemotoxylin as the counterstain. Note that the clustering in panel A indicates that reproduction has occurred. Bar = 5 μm.*

previously been shown to enhance excystation *(24).* This outcome was observed for oocysts incubated in apple cider prior to excystation (Table 2). The addition of acids to juices at lower concentrations most likely does not influence infectivity, and increasing amounts of acids were added to juices in this study before any inhibition of infection was observed. The effect of pH can be seen on an intracellular level, with sporozoite attachment to host cells being pH dependent to some degree, with an optimal pH for such attachment being 7.2 to 7.6 *(20).* Oocysts incubated in acidified juices may become permeabilized, and slight damage to the oocyst wall may reduce infectivity; however, oocysts were not observed to be visibly damaged with phase-contrast microscopy or with differential interference contrast microscopy (data not shown). Citric acid chelates cations and has been shown to exert an inhibitory influence against bacteria and molds in this manner *(4).* Citric acid, present in the largest quantities in orange juice and to lesser extents in the other juices, may inhibit infection by this mechanism, with sporozoites showing enhanced attachment to host cells in the presence of calcium, zinc, and magnesium *(20, 38).* It was also observed that the toxic response of cells to *Cryptosporidium* was reduced with a decrease in the calcium concentration of the environment and that the toxin may be mediated by calcium ions *(19).*

Effects of hydrogen peroxide on infectivity. Hydrogen peroxide inhibited *C. parvum* infectivity in the cell culture assay. Tables 2 and 3 show the inactivation of *C. parvum* by H_2O_2 in apple cider, orange juice, purple grape juice, and white grape juice as measured by excystation and infectivity, respectively. In contrast to the cell culture assay, which measures the potential of oocysts to complete their life cycle within the host, excystation is a measurement of the oocyst's response to a biochemical stimulus. The two assays are useful in interpreting the effects of treatments on oocysts. The results for the two tests are not necessarily correlative, because oocysts that excyst may not be able to complete their life cycle within the host. Excystation overestimates oocyst viability *(2),* particularly after chemical inactivation, compared with infectivity *(2)*; however, excystation provides insight into biochemical reactions that may occur within the oocyst wall. Infectivity as evaluated with the cell culture assay was completely inhibited by the in-

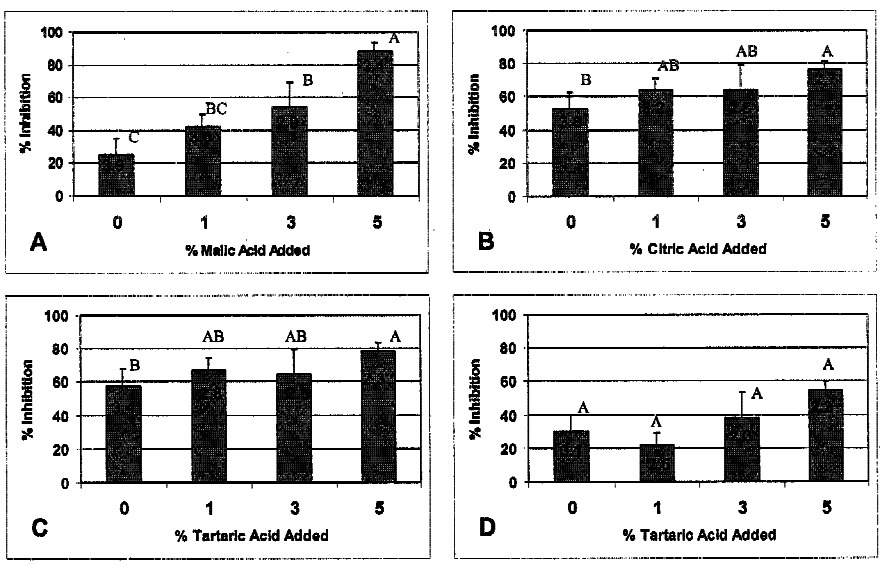

FIGURE 2. *Inhibition of* C. parvum *infectivity in HCT-8 cells. The dominant organic acid of each juice was added on a weight/volume basis to (A) apple cider, (B) orange juice, (C) purple grape juice, and (D) white grape juice. The percentage of inhibition is the average mean for at least three experiments. Error bars indicate the standard deviation. The number on each bar represents the average pH of the juice sample. Treatments with different letters in the same graph are statistically different* ($P \leq 0.05$).

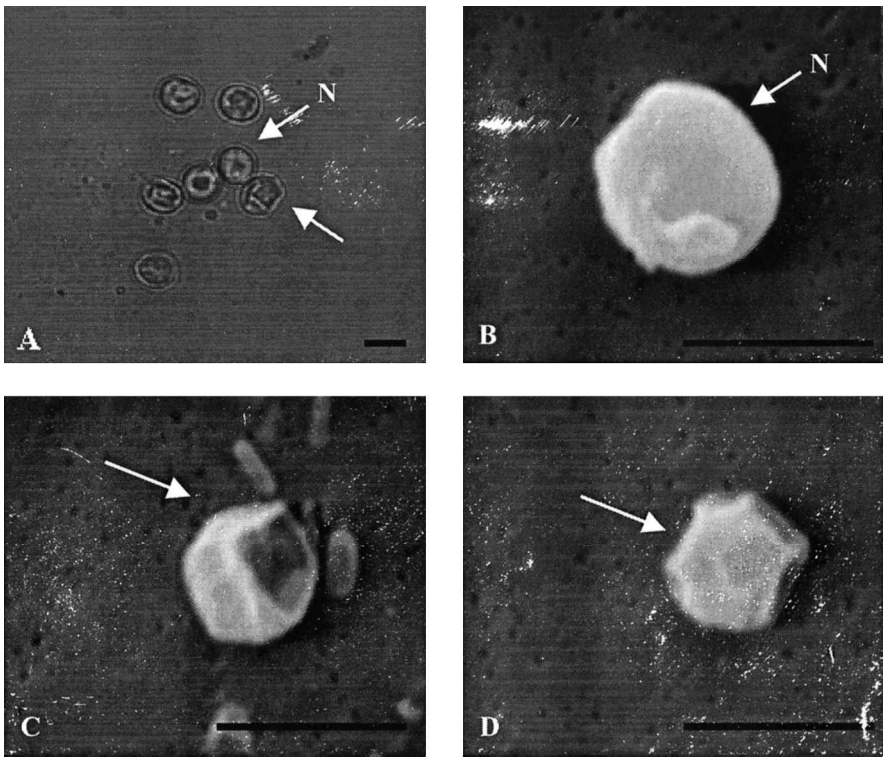

FIGURE 3. *Hydrogen peroxide damage to the* C. parvum *oocyst wall. Oocysts incubated with apple cider plus 0.03% H_2O_2 for 2 h at 5°C were observed with differential interference contrast microscopy (A) and SEM (B through D). Arrows may indicate areas of damage to oocyst walls, perhaps caused by radicals from H_2O_2 decomposition compared with oocysts that appear more normal (N) in shape (A and B). Bar = 5 μm.*

TABLE 2. *Inactivation of* C. parvum *oocysts in fruit juices treated with H_2O_2 as measured by in vitro excystation*

Sample (incubation time)	% excystation (SD)[a]	Log_{10} reduction in excystation[b]
HBSS	8.9 (8.4)	0.00
Apple cider		
Unamended (2 h)	84.9 (5.6)	+0.03
+ 0.025% H_2O_2 (≥6 h)	29.7 (5.1)	0.46
+ 0.03% H_2O_2 (1 h)	39.4 (6.3)	0.33
+ 0.03% H_2O_2 (≥2 h)	23.6 (4.3)	0.56
Orange juice		
Unamended (2 h)	88.0 (6.6)	0.00
+ 0.025% H_2O_2 (≥6 h)	24.7 (5.5)	+0.04
+ 0.03% H_2O_2 (1 h)	29.0 (6.7)	0.44
+ 0.03% H_2O_2 (≥2 h)	23.6 (4.0)	0.53
Purple grape juice		
Unamended (2 h)	88.0 (2.5)	+0.04
+ 0.025% H_2O_2 (≥6 h)	22.6 (4.0)	0.55
+ 0.03% H_2O_2 (1 h)	26.0 (6.3)	0.48
+ 0.03% H_2O_2 (≥2 h)	20.2 (5.0)	0.60
White grape juice		
Unamended (2 h)	93 (2.7)	0.00
+ 0.025% H_2O_2 (≥6 h)	23.0 (3.5)	+0.06
+ 0.03% H_2O_2 (1 h)	27 (3.0)	0.47
+ 0.03% H_2O_2 (≥2 h)	21.0 (3.3)	0.54

[a] Excystation rates are based on at least 50 oocysts which were scored as excysted or not excysted. Rates are calculated as number of excysted oocysts/total number of oocysts observed.

[b] The log_{10} reduction in excystation was determined on the basis of excystation rates for control oocysts treated with HBSS prior to excystation. Values represent averages for at least three experiments.

cubation of oocysts in apple cider plus 0.025% H_2O_2 for ≥6 h or in apple cider plus 0.03% H_2O_2 for ≥2 h. No signs of infection were observed for oocysts incubated in these juices, and the limit of detection with this assay is infection with ≥50 oocysts *(31).* The percentage of inhibition strongly correlates with the log_{10} excystation reduction as determined by the MPN method (>5 log units). It should be noted that identical cell culture assay results in terms of percentage of inhibition and log_{10} reduction were found for orange juice, white grape juice, and purple grape juice with 0.03% H_2O_2 added and for oocysts treated for 2 h prior to the infection of cell monolayers.

Excystation rates for these same treatments were low compared with those for unamended apple cider, for which oocysts still excysted but were unable to infect the cells. In unamended apple cider, sporozoites may be unable to infect cells because of DNA damage arising from reactions with hydroxyl radicals. Hydrogen peroxide readily breaks down into reactive oxygen species, including the reactive hydroxyl radical, which is well known to cause DNA damage by reacting with thymidine and other nucleic acids. Hydrogen peroxide has been shown to affect the calcium intake of the internal parasite *Leishmania donovani* both intracellularly and extracellularly, resulting in an apoptosis-like death *(9).* Das et al. *(9)* noted that the inhibitory action of H_2O_2 was in part due to a significant increase in calcium after a short incubation of parasite life stages in H_2O_2. Free radical production arising from H_2O_2 decomposition, along with changes in calcium ion concentration, is a common feature of programmed cell death, as observed for eukaryotic cells *(26).* As noted above, calcium is necessary for proper infection by *C. parvum (20, 38).* The influx of calcium ions from the environment may be another mechanism by which H_2O_2 inactivates *C. parvum* oocysts.

TABLE 3. *Inactivation of* C. parvum *oocysts in fruit juices treated with H_2O_2 as measured by cell culture infectivity*

Sample (incubation time)	% inhibition (SD)[a]	Log_{10} reduction in infectivity[b]
HBSS	0.00	0.0
Apple cider		
Unamended (2 h)	25.4 (3.8)	1.4
+ 0.025% H_2O_2 (≥6 h)	59.0 (10.3)	3.0
+ 0.03% H_2O_2 (1 h)	69.2 (9.7)	3.5
+ 0.03% H_2O_2 (≥2 h)	>99.0 (0)	>5.9
Orange juice		
Unamended (2 h)	52.5 (12.5)	2.8
+ 0.025% H_2O_2 (≥6 h)	>99.9 (0)	>5.9
+ 0.03% H_2O_2 (1 h)	77.5 (3.9)	4.4
+ 0.03% H_2O_2 (≥2 h)	>99.9 (0)	5.9
Purple grape juice		
Unamended (2 h)	57.6 (9.6)	3.0
+ 0.025% H_2O_2 (≥6 h)	>99.9 (0)	>5.9
+ 0.03% H_2O_2 (1 h)	75.3 (5.6)	4.1
+ 0.03% H_2O_2 (≥2 h)	>99.9 (0)	>5.9
White grape juice		
Unamended (2 h)	29.9 (13.8)	1.4
+ 0.025% H_2O_2 (≥6 h)	>99.9 (0)	>5.9
+ 0.03% H_2O_2 (1 h)	70.5 (10.0)	4.0
+ 0.03% H_2O_2 (≥2 h)	>99.9 (0)	>5.9

[a] A "greater than" sign indicates that no life stages were observed in the sample evaluated. For calculation purposes, it was assumed that one life stage was detected in the sample, and this value was used to calculate the log_{10} reduction in infectivity (*34*).

[b] The log_{10} reduction in infectivity was determined by an MPN-based analysis for cell culture infection with dilutions of 10^6, 10^5, and 10^4 oocysts. Percentages of inhibition and log_{10} reductions are not correlative. Values are averages for at least three experiments.

Since the addition of H_2O_2 did not change the pH of the amended juice samples compared with that of the control samples, changes in infectivity cannot be attributed to pH. Oocysts incubated in juices treated with these low levels of H_2O_2 were analyzed for possible reactivation. After treatment, oocysts were washed with HBSS and were found to remain noninfective after 2 weeks of storage at 5°C. Additionally, apple cider plus 0.03% H_2O_2 was found to be acceptable to >65% of panelists participating in a sensory analysis and using the in/out method *(29, 33)* (data not shown); thus, this treatment may offer an alternative to heat pasteurization. While taste was not affected, color as analyzed with a colorimeter for L*, a*, and b* values, and a slight decrease in yellow color was indicated.

Oocyst morphology. Differential interference contrast microscopy and SEM were used to evaluate *C. parvum* oocysts incubated in apple cider plus 0.03% H_2O_2 (Fig. 3). Some oocysts incubated in apple cider plus 0.03% H_2O_2 looked shriveled or injured. These oocysts lacked their characteristic round shape. With SEM, these alterations in the oocyst wall were still visible. This result may constitute evidence of a reaction with reactive oxygen species formed from the breakdown of H_2O_2.

Hydrogen peroxide has previously been shown to alter normal oocyst excystation *(32)*. Acid-fast staining was found to be greatly improved after treatment with 10% H_2O_2 *(12)*. In some cases, staining sensitivity was enhanced up to 40-fold after treatment. Sodium hypochlorite treatment also enhanced acid-fast staining, but to a lesser extent *(7)*. While H_2O_2 treatment presumably oxidizes or alters oocyst wall proteins, H_2O_2 treatment did not affect monoclonal antibody staining and does not seem to remove antigenic sites *(12)*. However, the formation of crystals after chlorine treatment interfered with antibody staining *(12)* and reduced levels of antibody detected by Western blot analysis and flow cytometry *(28)*.

Cryptosporidiosis is resistant to the majority of chemotherapeutic agents used to treat parasitic diseases, in part because the majority of chemotherapeutic agents have been directed against the organism in the intracellular or zoite stage rather than against oocysts *(21)*. This resistance may also be a reflection of the strength of the trilaminar oocyst wall, and while no treatment has been found to be effective in damaging the oocyst wall to date *(21)*, the relatively low concentrations of H_2O_2 used in this study appear to do so. Other treatments (such as freezing) that reduce infectivity and kill oocysts do not alter oocyst morphology *(15)*. While it is not clear whether the inactivation of *C. parvum* is based on one target (the oocyst) or multiple targets (four sporozoites) *(36)*, it is most likely that concentrations of H_2O_2 used in this study have a combined effect whereby hydroxyl radicals first oxidize and weaken the oocyst wall and then affect the DNA of sporozoites, rendering them noninfective. It is likely that H_2O_2 could be used alone or in combination with other inactivation methods such as ultraviolet light, freezing, or ozone to control the contamination of unpasteurized fruit juices by *Cryptosporidium*.

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